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14. ABSTRACT
Breast cancer is the most common non-skin cancer in women and the American Cancer Society estimates that there will be 215,990 new cases of invasive breast cancer and 40,110 deaths from MBC in the United States in 2004. Thus, patients with MBC who fail conventional therapies are candidates for clinical trials using novel therapeutic approaches, including immunotherapy. Dendritic cells (DC) are potent antigen-presenting cells that prime antitumor cytotoxic T lymphocytes against tumor-associated antigens and bacterial DNA oligodeoxynucleotides containing unmethylated CpG sequences (CpG DNA) further augment the immune priming functions of DCs. We hypothesize that CpG DNA-stimulated DCs will prime a more potent anti-tumor immune response than non-stimulated DCs. Our 3 specific aims are 1) to study the mechanism of antitumor immunity mediated by the vaccination of TS/A mammary tumor-bearing BALB/c mice with CpG DNA-stimulated DCs primed in vitro with necrotic TS/A cells, 2) to determine optimal conditions for CpG DNA stimulation and tumor priming of human DCs, and 3) to design a Phase I/II clinical trial of DC + CpG DNA therapy for breast cancer.

15. SUBJECT TERMS
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I. INTRODUCTION

Dendritic cells (DC) are potent antigen-presenting cells that prime antitumor immunity against tumor-associated antigens by cytotoxic T lymphocytes, and bacterial DNA oligodeoxy- nucleotides containing unmethylated CpG sequences (CpG DNA) can further amplify the immunostimulatory capacity of DCs. In this proposal we hypothesize that CpG DNA-stimulated DCs will prime a more potent anti-tumor immune response than non-stimulated DCs. In Aim 1 of this proposal, we test this hypothesis in a pre-clinical model of breast cancer using an established mammary tumor (TS/A) in syngeneic BALB/c mice. DCs are primed by necrotic tumor cells *in vitro* and then injected subcutaneously with CpG DNA in TS/A tumor-bearing mice. In Aim 2, as a prelude to a future clinical trial, we determine the optimal conditions for tumor priming and CpG DNA stimulation of human DCs. In this aim, we will develop optimal loading conditions of human DCs with tumor lysate derived from the MCF-7 human breast cancer cell line. In Aim 3, based on the pre-clinical data generated in Aims 1 and 2, we plan to undertake a Phase I/II clinical trial of immunization with autologous tumor-primed DCs and dose-escalated CpG DNA in patients with metastatic breast cancer.

II. BACKGROUND

Breast cancer is the most common non-skin cancer in women and the American Cancer Society estimates that there will be 211,240 new cases of invasive breast cancer and 40,410 deaths in 2005. Thus, patients with breast cancer who fail conventional therapies are candidates for clinical trials using novel therapeutic approaches, including immunotherapy.

APCs such as DCs are required to prime CTL against TAA in order to generate an effective antitumor immune response (reviewed in 1). DCs may be induced to synthesize important immunostimulatory cytokines such as interferon-alpha (IFN-α) and interleukin-12 (IL-12) (2, 3). In turn, IL-12 stimulates a T-helper-1 (Th1) immune response (3) important for the enhancement of CTL activity and induces the production of important antiangiogenic chemokines such as IP-10 and Mig (4). Numerous studies have demonstrated that DCs enhance the rejection of established murine tumors (5-11) using TAAs such as idiotypes, peptides, or tumor lysates, and such pre-clinical studies have formed the basis for DC-based immunizations of patients with cancer (12-18). Of note, a recent study by Avigan *et al* (19) has shown that a therapeutic DC-tumor fusion vaccine generated 2 partial responses and 1 stabilization of disease in 10 treated patients with MBC. Also, in a study completed at the University of Pittsburgh Cancer Institute (UPCI) using peptide-pulsed DCs in patients with advanced malignant melanoma, we have demonstrated 2 complete responses and 1 partial response in 28 patients, with no toxicity. Therefore, these encouraging results bode well for DC-based therapies of patients with MBC.

While DCs are potent APCs, efforts have been undertaken to further amplify their immunostimulatory capacity and bacterial DNA oligodeoxynucleotides (ODN) containing unmethylated CpG sequences (CpG DNA) provide such a stimulus (reviewed in 20). Thus, CpG DNA upregulates the expression of MHC and co-stimulatory molecules such as CD40 and CD86 by DCs (21), is a potent stimulator of DC-mediated CTL responses *in vivo* (22), stimulates DCs to secrete many potent immunomodulatory cytokines such as IL-12 (23, 24), TNF-α, and IFN-γ (Baar, unpublished data), and augments both natural killer (NK) cell and B cell activities (25-27). In our laboratory, we have demonstrated that the combination of tumor lysate-loaded DCs and CpG DNA was more effective mediating complete tumor regression in mammary tumor-bearing mice than either DCs or CpG DNA alone. Also, tumor-resistant mice successfully resisted a challenge with fresh mammary tumor cells, reflecting the generation of antitumor immune memory.

CpG 7909 is a clinical-grade ODN (Coley Pharmaceuticals) that has been tested as an immune adjuvant in numerous clinical trials (detailed in the Company's Investigator's Brochure and available

upon request). In a recent report of a Phase I clinical trial in patients with metastatic renal cell carcinoma (unpublished data), CpG 7909 was administered in dose tiers of 0.08, 0.12, 0.16, 0.36, and 0.54 mg/kg SC qw x 24 weeks. Two patients achieved a partial response at the 0.16 and 0.54 mg/kg dose tiers, and 14 patients had stabilization of disease by RECIST criteria. Also, there were no reported grade 3 or 4 toxicities. The study continues at a dose tier of 0.81 mg/kg. Therefore, by virtue of its stimulatory effect on many arms of the immune system and its activity as a single agent in patients with metastatic cancer, CpG DNA has a tremendous potential as an adjuvant in cancer immunotherapy.

III. BODY

Aim 1. To Study The Mechanism Of Antitumor Immunity Mediated By The Vaccination Of TS/A Tumor-Bearing Mice With CpG DNA-Stimulated DCs Primed In Vitro With Necrotic TS/A Cells.

A) CpG DNA Stimulates DCs to Produce Immunomodulatory Cytokines. We first studied the effect of CpG DNA (5'-TCCATGA_CG_TTCCTGATGCT-3', referred to as CpG-1S) on cytokine production by BALB/c DCs, since we were going to use BALB/c mice, a Th2-biased mouse model, for our therapeutic studies against a syngeneic BALB/c mammary tumor (TS/A). Thus, CD11c+ BALB/c DCs were generated from bone marrow precursors from BALB/c mice and grown for 7 days in supplemented RPMI medium containing 1000 u/ml of mGM-CSF and mIL-4 ("DC medium"). DCs were purified by magnetic cell sorting using anti-CD11c-microbeads (Miltenyi Biotech, CA). One million DCs were then plated in a 96-well plate in 200 µl of DC medium/well, and either 0, 0.1, 1, or 3 µM of CpG-1S or the negative control CpG-1NS were added to the DCs by gentle mixing. CpG ODNs were negative for endotoxin content. DCs were cultured for 24 hours @ 37°C, and supernatants were assessed by ELISA for p70 IL-12, IFN-γ, and TNF-α. We determined that BALB/c DCs were stimulated by CpG-1S to produce high levels of IL-12, IFN-γ, and TNF-α. Results revealed that peak IL-12 production (2400 pg/10^6 DC/24 hours) and IFN-γ production (21600 pg/10^6 DC/24 hours) by DCs was observed with 0.1 µM CpG-1S, whereas peak TNF-α production (1600 pg/10^6 DC/24 hours) was observed with 3 µM CpG-1S (Fig. 1). Therefore, CpG-1S was very effective at stimulating BALB/c DC, using cytokine production as a biological surrogate for DC activation.
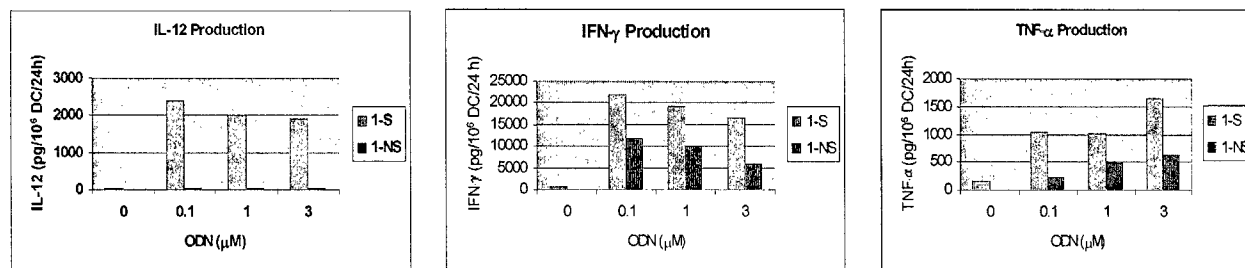

Fig. 1. CpG-1S Dose-Response of Cytokine Production by BALB/c DCs.

B) Therapy of An Established TS/A Murine Tumor in BALB/c Mice With TS/A Lysate-Loaded DCs and CpG-1S. As a "proof-of-principle" for combining DCs with CpG DNA for cancer therapy in a murine model of mammary carcinoma, 4 groups of BALB/c mice were inoculated in 2 independent experiments in the right flank with $5x10^4$ syngeneic TS/A mammary tumor cells and tumors were allowed to grow until they were palpable (~1 mm^2). Tumor-bearing mice were then vaccinated subcutaneously in the opposite flank twice, 7 days apart, with either (a) PBS, (b) TS/A lysate-loaded DCs alone ($5x10^5$ DCs), (c) CpG-1S alone (15 nmole, injection 1; 10 nmole, injection 2) or (d) DCs + CpG-1S. While none of the mice treated with either PBS or DCs alone survived, 100% of the mice

treated with DC + CpG-1S survived and were tumor-free, compared to only 37.5% of the mice treated with CpG-1S alone (P=0.0085) (Fig. 2). Thereafter, mice in both surviving treatment groups were rechallenged subcutaneously with 5×10^4 fresh TS/A tumor cells. All immune mice were able to reject the rechallenge with fresh TS/A tumor cells and remain tumor-free after 4 months of follow-up, suggesting the development of long-term antitumor immune memory after therapy. There is therefore a strong rationale for using tumor lysate-loaded DCs in combination with CpG DNA for cancer therapy.
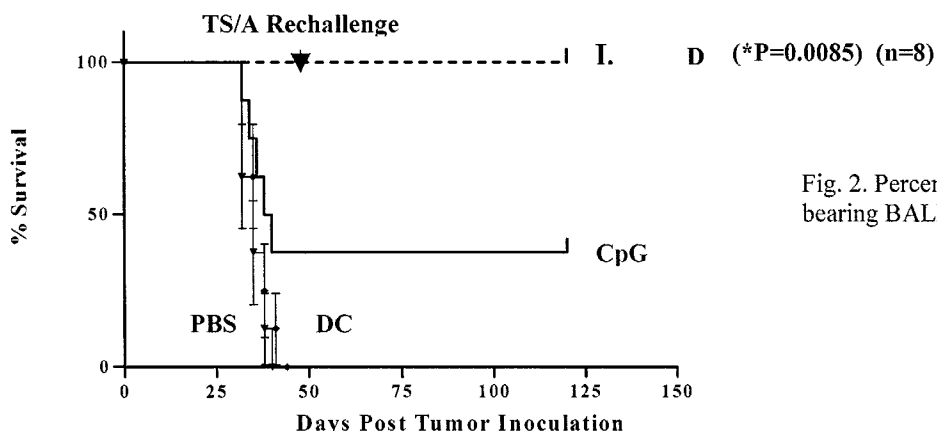
Fig. 2. Percent Survival of TS/A tumor bearing BALB/c mice.

C) DC + CpG-1S-Mediated Tumor Rejection Is Dependent On Both CD4+ And CD8+ T Cells.

In order to determine the immune mechanism for tumor rejection, 4 groups of BALB/c mice were inoculated in the right flank with 5×10^4 syngeneic TS/A mammary tumor cells and tumors were allowed to grow until they were palpable (~ 1 mm^2). Tumor-bearing mice were then vaccinated subcutaneously in the opposite flank twice, 7 days apart, with either (a) PBS, (b) TS/A lysate-loaded DCs (5×10^5) + CpG-1S (15 nmole, injection 1; 10 nmole, injection 2), (c) DCs + CpG-1S + anti-CD4 antibody, or (d) DCs + CpG-1S + anti-CD8 antibody. Both anti-CD4 and CD8 antibodies were administered at doses of 0.5 mg intraperitoneally on days –3, 0, 3, 7, and 10 post-therapy. Preliminary experiments revealed that this antibody dosing schema resulted in >95% systemic depletion of both CD4 and CD8 T-cells. As shown in Figure 3, systemic depletion of both CD4+ and CD8+ T cells resulted in near-total abrogation of the antitumor effect mediated by DCs + CpG-1S.
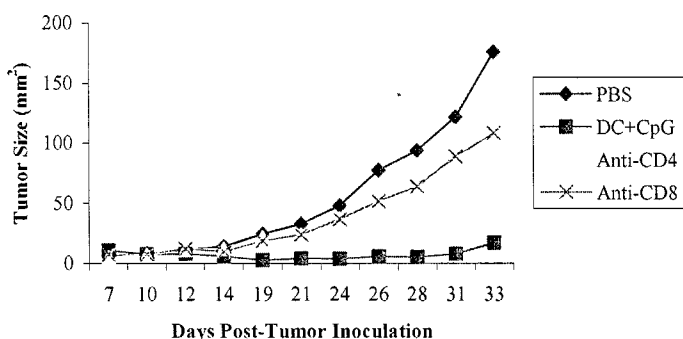
Fig. 3. Anti-CD4 and CD8 Blocking Studies

Aim 2. To Determine Optimal Conditions For CpG DNA Stimulation And Tumor Priming Of Human DCs.

Many DC-based human clinical therapy protocols utilize many cytokines (e.g. hGM-CSF and hIL-4) and maturation factors (e.g. LPS, IL-1, TNF-α, PGE2, poly I:C) to direct peripheral blood monocytes to differentiate into monocyte-derived DC (mDC). This process is cumbersome, expensive and generally requires 7 to 8 days of *in-vitro* culture to generate functional DCs. The endpoint of this grant

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proposal is to develop a simpler and more efficient DC clinical protocol to treat patients with metastatic breast cancer where CpG DNA will be used as the single, primary stimulus without using any other maturation factors to drive human peripheral blood DC precursors into mature DCs.

A subset of human DCs responsive to CpG DNA has been characterized by Krug *et al* (1). These plasmacytoid DC (pDC, CD123+) express the Toll-like receptor-9 (TLR9) which is involved in the recognition of CpG motifs. The blood dendritic cell antigen-4 (BDCA-4) is a unique pDC marker that allows for the isolation of these pDCs by elution from a BDCA-4 selection column. The authors demonstrated that a CpG DNA (5'-TCGTCGTTTTGTCGTTTT GTCGTT-3') supported the survival, activation (CD80, CD86, CD40, MHC class II), chemokine production (IL-8, IP-10) and maturation (CD83) of BDCA-4 selected pDC which were grown in culture medium containing hGM-CSF and hIL-3. In another study, Gursel *et al* (2) used another CpG DNA (5'-GTGCATCGATGCAGGGGGG-3') to stimulate human peripheral blood monocytes to mature into functionally active DC over 2-4 days. The transition from monocyte to DC was characterized by the up-regulation of CD83, CD86, CD80, CD40 and the down-regulation of CD14 in serum- and cytokine-free medium (XVIVO) containing only CpG DNA as the maturation factor. The differentiation of these monocytic DC (mDC) precursors to functional DCs was mediated by pDC present at low frequency in the peripheral blood sample, which responded to the CpG DNA by secreting IFN-alpha which, in turn, induced the maturation of the mDCs. The 2 major advantages of this latter approach compared to the former is that, first, no column selection procedure is required to isolate DCs, thereby significantly increasing the yield of mature DCs and, second, no cytokines (GM-CSF or IL-4 or IL-3) are required in the growth medium to sustain DC growth and maturation. We were therefore interested in doing a head-to-head comparison of the ability of these 2 CpG DNAs to stimulate human DCs.

OBJECTIVES

The objectives of this task were to:
1. Test the 2 above-mentioned CpG DNAs on both pDC and mDC and compare their effect on DC maturation (e.g. upregulation of MHC Class II, CD40, CD86, etc).
2. Test MCF-7 tumor lysate uptake by DCs after CpG stimulation and determine whether DC maturation markers are altered by tumor lysate loading.
3. Use autologous DC-primed T cells in an *in-vitro* cytotoxicity assay against MCF-7.

MATERIALS AND METHODS

CpG DNAs. 1: 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3' (used for pDC maturation or as indicated); 2: 5'-GGTGCATCGATGCAGGGGGG-3' (used for mDC maturation or as indicated).

Generation of pDC. Peripheral blood mononuclear cells (PBMC) were isolated form health human donors by Ficoll gradient separation. PDC were selected from PBMC by anti-BDCA-4 conjugated magnetic beads. pDC eluted from separation column were cultured in the presence of hGM-CSF and hIL-3 for 1 day (Fig. 4).

Generation of mDC. PBMC were separated by Percoll gradients into lymphocyte and monocyte sections. Monocytes were cultured in XVIVO® medium in the presence of CpG DNA but without any other cytokines or maturation factors. The rationale for the use of unselected mDCs is that these aliquots contain both pDC and mDCs. The addition of CpG DNA to the growth medium stimulates the pDCs to produce IFN-α which, in turn, stimulate the maturation of the mDCs in the culture (Fig. 4).
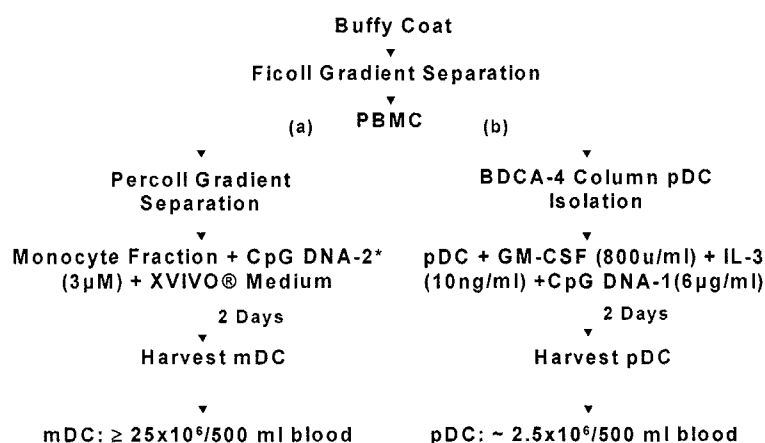
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                    Buffy Coat
                        ▼
            Ficoll Gradient Separation
                        ▼
              (a)    PBMC    (b)
         ▼                           ▼
   Percoll Gradient          BDCA-4 Column pDC
     Separation                  Isolation
         ▼                           ▼
Monocyte Fraction + CpG DNA-2*   pDC + GM-CSF (800u/ml) + IL-3
  (3µM) + XVIVO® Medium        (10ng/ml) +CpG DNA-1(6µg/ml)
       2 Days                       2 Days
         ▼                           ▼
    Harvest mDC                  Harvest pDC


         ▼                           ▼
mDC: ≥ 25x10⁶/500 ml blood    pDC: ~ 2.5x10⁶/500 ml blood
```

Fig. 4. Preparation of pDC and mDC from human peripheral blood

DC Surface Markers. Markers were detected through fluorescent-conjugated antibody to CD80, CD86, CD83, CD40, class II and etc by flow cytometer.

Tumor Antigen Loading. The human breast cancer cell line (MCF-7) lysate was prepared through repeated freeze and thaw cycles and was added overnight to the DC preparation in a 1:1 ratio of tumor cells to DCs. The next day, the loaded DCs were washed and labeled for FACS analysis.

Tumor Labeling. To monitor tumor uptake by DC, tumor cells were stained with DiI before loading onto DC.

Cytotoxicity Assays. Monocytes isolated from PBMC were grown for 1 day in XVIVO medium containing CpG DNA. The breast cancer cell line MCF-7 was lysed by repeated freezing and thawing. Tumor lysate was added to DC at the ratio DC: Tumor = 1 :1 for 1 day. After harvesting the tumor-pulsed DCs, they were used to prime autologous T cells (PBMC) at the ratio T:DC = 10:1. Il-2 (20 u/ml) was added to the medium on Day 5. T cells were restimulated every week with the CpG-treated DC. CTL were harvested after 3 stimulations and used as effectors against MCF-7 in an LDH cytotoxicity assay (Promega).

RESULTS

1.   Test CpG DNA On Both pDC And mDC And Compare Their Responses By DC Markers.

We observed that both CpG DNAs were equally effective in upregulating both pDC- and mDC-associated cell-surface markers such as MHC class II, CD40, CD80 and CD83. Maximum upregulation of these cell-surface markers was usually seen after 48 hours of DC culture (Figs. 5a and 5b).

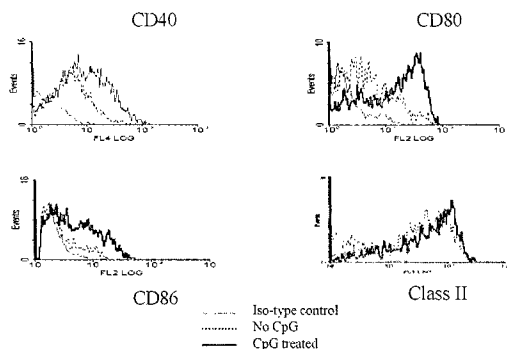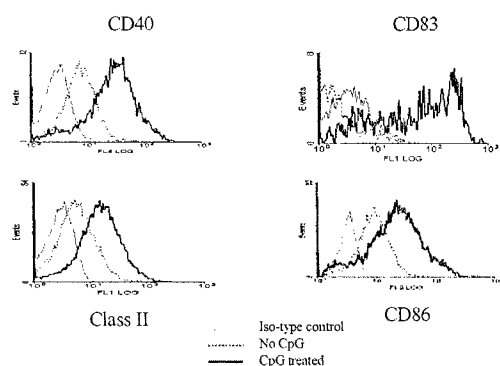a) CpG DNA-1 Upregulates pDC Marker Expression

CD40   CD80

CD86   Class II
          .... Iso-type control
          .... No CpG
          ——— CpG treated

Fig 5. Upregulation of Maturation Markers
by CpG DNA in a) pDC and b) mDC

b) CpG DNA-2 Upregulates mDC Marker Expression

CD40   CD83

Class II   CD86
          Iso-type control
          .... No CpG
          ——— CpG treated

Since CpG DNA-1 is commercially available and CpG DNA-2 is not, and since CpG DNA-1 has been tested for its ability to induce the maturation of pDC but not mDC in bulk peripheral blood, we tested whether CpG DNA-1 could induce the maturation of mDC. Thus, peripheral blood monocytes were grown in XVIVO medium containing 3 μM of CpG DNA-1. As shown in Fig. 6, this CpG DNA was equally effective in stimulating the maturation of both pDCs and mDCs.

CpG DNA-1 Enhances DC Markers Similarly on mDC and pDC

M-DC   pDC

CD40

Class II

Results were obtained in parallel isomer experiments
          ——— Iso-type control
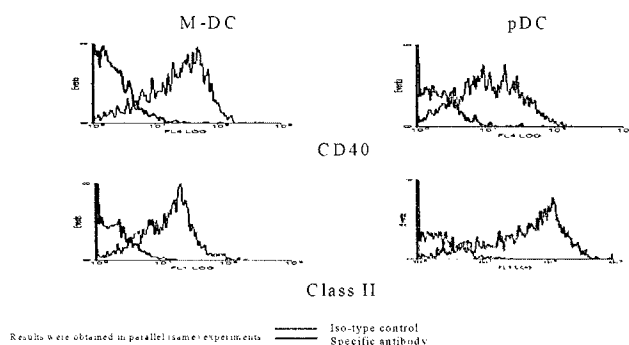   ——— Specific antibody

Fig. 6. CpG DNA-1 Is Equally
Effective In Inducing The
Maturation Of pDCs and mDCs

Therefore, we elected to use CpG DNA-1 in all of our subsequent experiments, as detailed below.

2. Test MCF-7 Tumor Lysate Uptake By DCs After CpG Stimulation And Determine Whether DC Maturation Markers Are Altered By Tumor Lysate Loading.

We observed that mDCs were efficient in taking up lysate from the MCF-7 human breast cancer cell line and that cell-surface DC marker expression was not reduced after tumor lysate loading (Fig. 7).
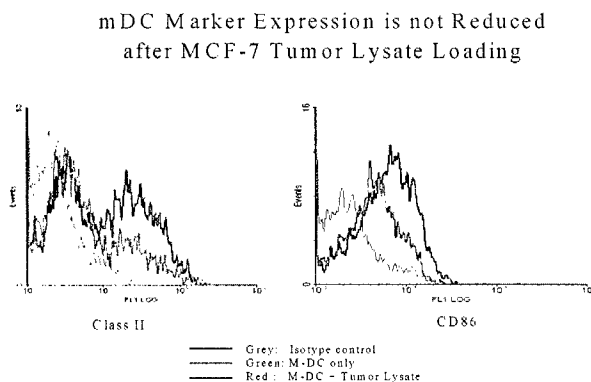

mDC Marker Expression is not Reduced
after MCF-7 Tumor Lysate Loading

Class II   CD86

Grey: Isotype control
Green: M-DC only
Red : M-DC - Tumor Lysate

Fig. 7. mDC Marker Expression is not Reduced after MCF-7 Tumor Lysate Loading

3. Use Autologous DC-Primed T Cells In An In-Vitro Cytotoxicity Assay Against MCF-7.

When autologous CTLs were primed by mDCs loaded with MCF-7 tumor lysate, we observed a dose-dependent killing of MCF-7 (Fig. 8).
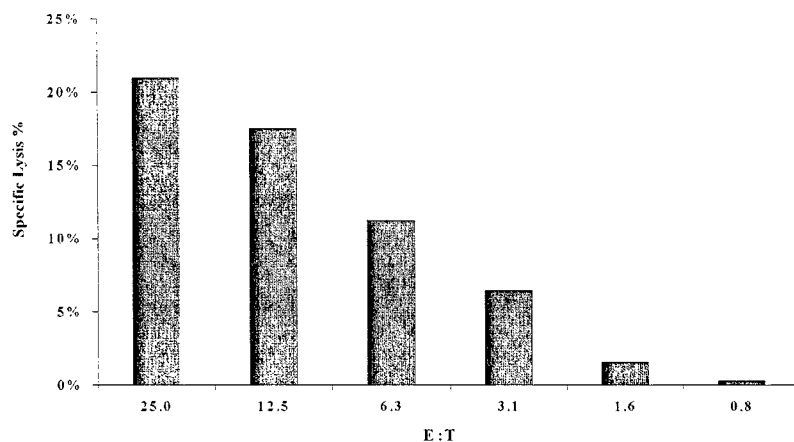

Fig. 8. Killing of MCF-7 Human Breast Cancer Cells by T Cells Primed with CpG DNA Treated DC Pulsed with MCF-7 Tumor Lysate

III. KEY RESEARCH ACCOMPLISHMENTS

Thus, we conclude that:

1. TS/A tumor lysate-loaded DCs and CpG DNA are an effective therapy for an established TS/A tumor in BALB/c mice.
2. We can generate, within 48 hours, a high number of functional DCs from bulk PBMCs using the commercially-available CpG DNA-1 as the sole DC maturation stimulus. A significant cost-saving is derived from the fact that expensive exogenous cytokines are not required for DC growth and there is no requirement for a column-selection methodology to isolate the activated DCs.
3. Human DCs stimulated by CpG DNA-1 and loaded with lysate from the MCF-7 human breast cancer cell line prime an effective cellular response against MCF-7. This is an important finding

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because it demonstrates that DCs that have been "matured" by CpG DNA still have the capacity to take up tumor antigen for processing and presentation to cytotoxic effector cells.

Therefore, based on the pre-clinical data detailed above, we have designed a Phase I/II clinical trial of DC + CpG DNA for women with metastatic breast cancer, as detailed below in Aim 3.

Aim 3. To Undertake A Phase I/II Clinical Trial Of Immunization With Autologous Tumor-Primed DCs And Dose-Escalated CpG DNA In Patients With Metastatic Breast Cancer.

A. Research Design And Methods

1) Rationale and Hypothesis

We hypothesize that CpG DNA-stimulated DCs will prime a potent anti-tumor immune response *in vivo* and we will test this hypothesis in a Phase I/II clinical trial of immunization with autologous tumor lysate-primed DCs and dose-escalated CpG DNA in patients with MBC. The rationale for this clinical trial derives from our pre-clinical data showing that (a) tumor-lysate loaded DCs and CpG DNA are effective in mediating the regression of an established murine mammary tumor *in vivo*, and (b) CpG DNA-1-stimulated human DCs can prime an anti-tumor cellular response against the human mammary carcinoma cell MCF-7.

NB: Since the trade designation for CpG DNA-1 by Coley Pharmaceuticals is CpG 7909, CpG DNA-1 will hereby be designated as CpG 7909 for the purposes of this clinical trial.

2) Specific Aims

The Phase I portion of the clinical trial will determine the starting dose of CpG 7909 for the Phase II trial, to be followed by the Phase II trial which will evaluate the efficacy of the treatment under consideration. The specific aims of this trial are:
1. Phase I:
   a) To study the safety and establish a starting dose for CpG 7909 in combination with the autologous DC vaccine in women with MBC.
   b) To characterize the immunologic response in patients who have received the DC + CpG 7909 vaccine.
2. Phase II:
   a) To measure clinical tumor response to the DC + CpG 7909 vaccine.
   b) To characterize the immunologic response in patients who have received the DC + CpG 7909 vaccine.

3) Patient Selection

- Only patients with MBC are eligible.
- Patients must have tumor cells accessible to harvest by either biopsy, resection, or from malignant effusions for the DC vaccine preparation.
- Patients must have radiologically-measurable MBC after the tumor cell harvest.
- Patients must have reactivity to at least 1 of 3 PCI skin tests (see below).
- Patients must have fully recovered from surgery, and must not have received any chemotherapy, radiotherapy, hormonal therapy, or immunotherapy within 4 weeks preceding vaccination.

- No more than 2 prior therapeutic regimens for MBC are permitted (excluding radiation therapy).
- Patients with treated and stable brain metastases are eligible.
- Patients requiring therapy with steroids are not eligible.
- Patients must have an expected survival of at least three months.
- Patients must have an ECOG Performance Status (PS) of ≤ 1 (Karnofsky PS ≥ 80).
- Patients must have the following initial and subsequent pretreatment laboratory parameters:
  - ◇Granulocytes: $\geq 1,500/mm^3$
  - ◇Lymphocytes: $\geq 700/mm^3$
  - ◇Platelets: $\geq 100,000/mm^3$
  - ◇Serum Creatinine: ≤ 2.0 mg/100 ml
  - ◇Serum Bilirubin: ≤ 2.0 mg/100 ml
- Patients must be able to give written informed consent.
- Patients must not be pregnant or lactating.

4) PCI Skin Test

- Following evaluation and meeting the eligibility criteria noted above, the multitest PCI skin test with antigens for cellular hypersensitivity will be applied in initial patient screening (≤ 4 weeks prior to 1st DC vaccination).
- Each test contains a disposable applicator which is preloaded with 1 of 3 antigens for i.d. injection, including tetanus, tuberculin, Candida, and a normal saline solution control.
- Induration will be measured at 24 and 48 hours by a Study Investigator or an assigned representative (Nurse, PA, NP) and must measure ≥ 2 mm.
- Patients must have reactivity to at least 1 of the 3 antigens to be eligible for entry into the clinical trial.

5) DC Preparation

Eligible patients with MBC will undergo a tumor cell harvest and a leukapheresis for DC generation by the Immune Monitoring and Cellular Processing Laboratory (IMCPL) of the University of Pittsburgh Cancer Institute (UPCI). DCs will be grown for 6 days in complete medium containing 1000 u/ml of both hGM-CSF and hIL-4. Lysate from autologous breast cancer cells will then be co-cultured overnight with the DCs. Next day, the lysate-loaded DCs will be washed and then matured for 24 hours by adding 10 mg/ml IL-1β, 1000 U/ml IL-6, and 10 ng/ml TNF-α to the co-culture. Next day, DCs will then be washed, counted, tested for sterility, and be split into 3 aliquots: ⅓ of the DC vaccine will be used for the 1st DC vaccination (Day 1); ⅓ of the DC vaccine will be frozen and then thawed and cultured for 1 day prior to the 2nd DC vaccination (Day 8); and ⅓ of the DC vaccine will be frozen and used for the *in-vitro* assays detailed below.

6) DC + CpG 7909 Vaccination

For each scheduled day of vaccination for each patient, DCs will be counted, equally distributed among 8 syringes (i.e., 7 syringes containing the tumor-loaded DCs and 1 syringe containing Hepatitis-B Surface Antigen (HBSA)-pulsed DCs as a positive-control) and mixed with the scheduled dose of CpG 7909 (see dosing schema below). Each patient will be administered the DC + CpG 7909 vaccine intradermally (i.d.) on Days 1 and 8 at eight different sites using the right and left anterior chest, proximal arms, right and left lower abdomen and proximal thighs for each vaccination. All patients will be treated in the UPCI-affiliated General Clinical Research Center (GCRC).

Phase-I Dosing of CpG 7909. Patients will receive the autologous tumor lysate-loaded DCs admixed with the CpG 7909 adjuvant in 4 dose-tiers of CpG 7909 (escalated in 2-fold dose increments; see table below) that emulate the dose range of CpG 7909 administered in the Phase I trial of patients with metastatic kidney cancer (i.e., 0.08 – 0.81 mg/kg). For each vaccination, each of the 8 syringes will be loaded with 1/8th of the total scheduled dose of CpG 7909.

Tier	Day-1 (mg/kg)	Day-8 (mg/kg)
1	0.1	0.1
2	0.2	0.2
3	0.4	0.4
4	0.8	0.8

Phase II Dosing of CpG 7909. The starting dose for CpG 7909 in the Phase II portion of the trial will the maximum dose determined in Phase I. Similarly to the Phase I trial, each patient will receive 2 vaccinations 7 days apart. For each vaccination, each of the 8 syringes will be loaded with 1/8th of the total scheduled dose of CpG 7909 and administered i.d at eight different anatomical sites, as described above.

Additional Vaccinations. If a patient has either a partial response or stable disease by RECIST criteria, the patient will be eligible for another course of DC + CpG 7909 vaccination. However, additional vaccinations will require a repeat leukapheresis to generate DCs and a repeat harvesting of tumor cells. If a patient has a complete response, no further vaccinations will be administered and the patient will be followed up at 3-month intervals, as detailed in the appended protocol. If a patient has progressive disease, no further vaccinations will be administered.

B. Endpoints

1) Clinical Endpoints

Seven weeks after the 2nd vaccination, patients will be restaged radiologically to score for clinical response using RECIST criteria.

2) Immunologic Endpoints

- ELISPOT Assay. Peripheral blood (60 ml) will be drawn one week before the first vaccination and 7 weeks after the second vaccination, the latter to coincide with the planned radiological assessment for clinical response. Peripheral blood lymphocytes (PBL) (20×10^6) will be incubated in tissue culture tubes in 4 ml RPMI-1640, 10% heat-inactivated human AB serum, antibiotics, and 50 IU/ml rhIL-2. After 6 days, T cells will be evaluated in ELISPOT assays. Pre-and post-vaccination PBL (at least 10^7) will be separated into CD4+ and CD8+ fractions by selection on immunobeads. The enriched fractions will be counted and tested by flow cytometry to determine the percent of CD8+ and CD4+ T cells in each. The enriched fractions will be tested in 20h ELISPOT assays performed in triplicate wells, each containing 50,000 cells/well. The ratio of T cells to DC will be 10:1. The assays will be formatted as follows: 1) CD8+T cells alone; 2) CD4+T cells alone; 3) CD8+T cells + irradiated tumor alone; 4) CD4+T cells + irradiated tumor alone; 5) CD8+T cells + unprimed DC; 6) CD4+T cells + unprimed DC; 7) CD8+T cells + tumor-loaded DC; 8) CD4+T cells + tumor-loaded DC; 9) CD8+T cells + Hepatitis B sAg; 10) CD4+T cells + Hepatitis B sAg; 11) CD8+T cells + DC + Hepatitis B sAg; and

12) CD4+T cells + DC + Hepatitis B sAg. After incubation, the plates will be developed, and the numbers of spots in experimental and control wells will be counted, using a Zeiss image analysis system. The ELISPOT assay results will be expressed as the difference between the mean number of spots in the experimental wells - mean number of spots in the control wells, or as the frequency of IFN-γ-secreting cells in total cells plated per well. The final results will be expressed as the frequency of CD4+ and CD8+ T cells responding to cryptic tumor antigens in the population tested. The expectation is that patients responding to the DC vaccination will demonstrate a significant difference in the frequency of responding CD4+ and CD8+ T cells before and after DC vaccination.

- <u>Regulatory T Cells</u>. From the blood obtained above, we also propose to monitor for the presence and changes in absolute numbers of Tregs prior to and after vaccination. This will be accomplished by multicolor flow cytometry, gating for CD4+ T lymphocytes which are CD25^{bright} and also express one or more of the following markers: Foxp3, GITR and CTLA-4. These markers will be assessed in CD4+CD25^{bright} T cells following their permeabilization with buffered saponin and staining with the following Abs: Anti-Foxp3-FITC: eBioscience, #11-5779-73, Lot #E013534, clone hFOXY; Anit-GITR-FITC: R&D Systems Inc., FAB689F, Lot #LAU014071, mouse mAb (IgG1); Anti-CTLA4-PE: Immunotech, IM2282, clone BNI2, Lot #09, mouse mAb (IgG2a). The IMCPL has been optimizing flow cytometry for these markers, and is now using this methodology for quantification of Tregs in the circulation of patients with cancer (Schaefer C, et al, Br. J. Cancer, In Press, 2005). The expectation is that the absolute numbers of Tregs detectable in the circulation will decrease after successful vaccination and will correlate with positive anti-tumor immune responses measured in ELISPOT assays.

C. STATISTICS

This is a combined Phase I/II clinical trial to find a starting dose of CpG 7909 and to evaluate the efficacy of the combination of CpG 7909 and autologous DCs at the selected dose of CpG 7909. In the Phase I portion, the dose of CpG 7909 will be escalated in four tiers to 0.8 mg/kg. The safety of the first 3 dose-tiers will be evaluated by treating 3 patients at each tier and observing patients for toxicity for 2 weeks before treating a new patient at the next higher dose. If a grade III/IV toxicity (NCI Common Toxicity Criteria) is observed at any dose tier, the dose escalation scheme will immediately switch to the conventional Phase I design for finding MTD (3 to 6 patients per tier with de-escalation). If grade III/IV toxicities are observed, the MTD will be defined as the highest dose with fewer than 1/3 of patients experiencing a grade III/IV toxicity. If no grade III/IV toxicities are observed, 0.8 mg/kg CpG 7909 will be chosen as the starting dose for the Phase II portion of the study without declaring a MTD.

The Phase II trial will then commence at the starting dose determined in Phase I. The sample size will be based upon the power of one sample binomial test. It is assumed that an objective response rate of 10% would be considered too low to warrant further study of this combination. We would like to have sufficient power to detect a clinical response rate of 30%. Eighteen patients will provide 80% power to detect this improvement using an exact one-tailed one sample binomial test at $\alpha = .10$. Thus, 12 patients will be required for the Phase I dose finding evaluation and 18 patients are needed for Phase II which includes the last 3 patients treated on the final Phase I dose. Therefore 27 patients are required for the complete study. Data analysis on both the Phase I and Phase II portions of the study will characterize immunologic response. The immunologic measurements to be evaluated include pre- and post-vaccination T cell reactivity as measured by the ELISPOT assay and the number of CD4+CD25+ T regulatory cells. PBMC samples for immunologic monitoring will be obtained at baseline and 7 weeks post-vaccine. Estimates of changes in T cell reactivity and the number of T

regulatory cells and associated confidence intervals will be calculated. Vaccine-mediated effects will be examined via either a signed rank test for continuous data or McNemar's test for binary data. Since this is a vaccine trial with a variable number of tumor-pulsed dendritic cells available for the vaccine, immunogenic and clinical responses will be compared to the final number of delivered DCs in the vaccine as well as any phenotypic or functional characteristics of the vaccine.

III. REPORTABLE OUTCOMES

One or 2 papers will be written to describe the data generated in Aims 1 and 2. Furthermore, a third paper will be generated encompassing the data derived from the planned clinical trial

IV. CONCLUSION

Based on our data, we conclude that CpG DNA-stimulated DCs prime a potent anti-tumor immune response.

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